Clinical isolates of GB virus type C vary in th ir ability to persist and replicate in periph ral blood mononuclear cell cultur s

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ABSTRACT

GB virus C/hepatitis G virus (GBV-C) replication *in vitro* is inefficient and inconsistent. In this study, clinical isolates of GBV-C were evaluated using peripheral blood mononuclear cell(PBMC) based culture methods. Isolates varied consistently in their ability to persistently replicate, and yield increased in cells grown without PHA/IL-2 stimulation. The deduced polyprotein sequence of an isolate that replicated well was determined (GenBank AY196904) and compared to 20 full-length GBV-C sequences. Fourteen of the sixteen unique amino acid polymorphisms identified were in the coding regions for non-structural proteins associated with interferon resistance and RNA replication. These data indicate that clinical GBV-C isolates vary in their ability to persist in culture, do not require PHA/IL-2 stimulation, and that sequence variability in key regulatory regions may affect growth in PBMC cultures. Since GBV-C appears to inhibit HIV replication in a co-infection model, these studies should facilitate determination of the mechanism of this interaction.

Keywords: GB Virus; Hepatitis G; In Vitro replication; polyprotein;

INTRODUCTION

GB Virus type C/hepatitis G virus (GBV-C) infection of humans is common; approximately 2% of healthy blood donors, 15% of HCV positive individuals, and 25% to 40% of HIV-infected people have GBV-C RNA in their serùm (Alter et al. 1997; Dawson et al. 1996; Feucht et al. 1997; Gutierrez et al. 1997; Linnen et al. 1996; Tacke et al. 1997). GBV-C is the most closely related human virus to hepatitis C virus (HCV), and infection with either GBV-C or HCV may result in persistent infection and prolonged viremia. Approximately 25% of HCV infections are spontaneously cleared by the host immune system (reviewed in Hoofnagle 1997). In contrast, an estimated 50% to 75% of GBV-C infections are cleared (reviewed in Alter 1997). HCV infection frequently results in chronic, progressive liver disease; however, GBV-C infection has not been convincingly associated with any disease (Alter 1997;Alter, et al. 1997).

Several studies have found that people coinfected with GBV-C and human immunodeficiency virus (HIV) appear to have longer AIDS free survival than HIV-infected people without GBV-C (reviewed in George et al. 2002b). In addition, GBV-C infection in HIV-positive people was associated with higher baseline CD4+ T cell counts, lower plasma HIV RNA concentration, and better response to anti-retroviral therapy (Tillmann et al. 2001; Xiang et al. 2001; Yeo et al. 2000). The survival benefit of GBV-C infection in HIV-positive people was most consistently demonstrated in individuals with GBV-C viremia; however, people with antibodies to the GBV-C envelope glycoprotein (E2) and who cleared GBV-C RNA also had improved survival when compared with HIV-infected people without evidence of prior or active infection with GBV-C (Tillmann, et al. 2001; Yeo, et al. 2000).

The mechanism by which GBV-C infection alters HIV disease progression is not established. Tillmann et al. reported an inverse correlation between HIV viral load and GBV-C plasma RNA concentration, suggesting an antagonistic effect of GBV-C on HIV replication. GBV-C was shown to replicate *in vitro* in peripheral blood mononuclear cells (PBMCs) (Fogeda et al. 1999), and a GBV-C infectious clone replicated *in vitro* in the CD4+ T cell subset of cultured PBMCs (Xiang et al. 2000). *In vitro* coinfection of PBMCs with GBV-C and HIV demonstrated diminished HIV replication (Xiang, et al. 2001), suggesting a direct inhibitory effect of GBV-C on HIV replication. GBV-C infection of PBMCs did not result in altered expression of the HIV receptors CD4, CCR5, or CXCR4 in the first 48 hrs post-infection, nor in decreased PBMC viability or protein syntheses during the first week post-infection when compared to mock-infected controls (Xiang, et al. 2001). Thus GBV-C did not appear to alter HIV entry or lead to decreased HIV replication due to cellular toxicity. Furthermore, the amount of inhibition of HIV replication was similar when the two viruses were added to PBMCs simultaneously or when the HIV infection preceded GBV-C (Xiang, et al. 2001). The inhibitory effect of GBV-C infection was more pronounced when GBV-C infection preceded HIV, suggesting that GBV-C replication may induce a cellular factor or factors that inhibited HIV replication (Xiang, et al. 2001).

Fogeda et al used plasma from a person with GBV-C viremia to infect human PBMCs (Fogeda, et al. 1999). Distinct quasispecies of GBV-C were identified in culture supernatant fluids following 30 days in culture that differed from the predominant quasispecies present in the plasma used to initiate infection, suggesting selection of a culture-adapted species (Fogeda et al. 2000). In this study, we evaluated clinical

isolates of GBV-C to determine if they differed in their ability to replicate *in vitro*, and to further characterize GBV-C replication and persistence in human PBMC cultures. We found considerable variation in the persistence and amount of GBV-C replication in PBMCs, suggesting that both viral determinants and donor cell variability were important in GBV-C replication. In addition, we determined the nucleotide sequence of the entire coding region of a clinical GBV-C isolate that replicated for $\geq$ 60 days in PBMC cultures. We compared the sequence of this isolate to the sequence of the isolate we used to prepare the GBV-C infectious clone, which did not replicate in PBMC cultures beyond 14 days, and to 20 full-length GBV-C sequences in GenBank.

RESULTS

GBV-C isolates from 12 HIV-positive people who had GBV-C RNA detected in blood on at least two prior occasions were analyzed in infectivity experiments. These GBV-C-HIV coinfected people had a mean CD4+ T cell count of 305 cells/mm^3 (range 64-885 cells/mm^3), mean CD8+ T cell count of 1324 cells/mm^3 (range 540-2588 cells/mm^3), and mean ALT values of 36 U/L (range: 10-147 U/L) (Table 1). Four of the patients had detectable HIV RNA in plasma at the time of PBMC collection, and 3 patients were HCV antibody positive. The mean age was 45 years (range 28-60 years); 10 patients were male, 1 patient was African-American, and 11 patients were Caucasian (data not shown). Ten patients reported sexual risk factors for HIV transmission and two reported IV drug use (data not shown). These 12 patients were representative of our clinic population.

Plasma GBV-C quantification and characterization: All 12 patients had GBV-C RNA detected in plasma on the day of PBMC collection, ranging from 22,950 genome equivalents (GE)/mL to 5.6 $x10^8$ GE/mL using real-time PCR (Table 2). Semiquantitative nested RT-PCR reactions performed using serial dilutions of patient plasma RNA as template confirmed the GBV-C RNA concentrations within an order of magnitude (Table 2). Eleven of 12 patients were infected exclusively with GBV-C genotype 2; whereas patient 3 had PCR products using primers specific for both genotypes 1 and 2. As described by others, 3 patients (1, 6, and 7) had detectable GBV-C E2 antibody in the same plasma sample used to quantitate RNA and perform infectivity studies (data not shown; (Tillmann, et al. 2001).

GBV-C Infectivity Studies: Phytohemagluttinen (PHA) and Interleukin-2 (IL-2) stimulated PBMCs from GBV-C/HIV coinfected patients were cultured with and without supplementation with PBMCs obtained from healthy donors (HD), and HD PBMCs were infected with GBV-C RNA-positive plasma (Figure 1). GBV-C RNA was detected in both supernatant and cultured PBMCs from all 12 patients; however, persistence of GBV-C RNA in culture varied significantly between patients (Figure 2A). PBMC cultures from patients 8 through 12 consistently had GBV-C RNA detected in culture supernatant and PBMCs for > 7 days, while patients 1-6 did not have GBV-C RNA detected in culture after 7 days. GBV-C persistence in culture was not enhanced by coculture with HD PBMCs (Figure 2B and C). GBV-C RNA detection in PBMC cell lysates was consistent with culture supernatant findings (data not shown). The concentration of GBV-C RNA in GBV-C RNA-positive donor PBMCs and in healthy donor cells infected with GBV-C isolates was low, ranging from 1 genome equivalent per 1500 to 200,000 PBMCs (data not shown). Studies are underway to develop methods to quantify the percent of PBMCs productively infected with GBV-C. No difference in cell viability was noted between different patients' PBMC cultures or between PBMCs cultured with and without HD PBMCs with the exception of patient 3, whose cultured PBMCs were no longer viable after day 7 (probably due to the high HIV inoculum (Table 1). When pooled stimulated HD PBMCs were infected simultaneously with 20 μl of each patient's plasma, patients 8 and 10-12 reproducibly had GBV-C RNA detectable in supernatants for 14 days or longer, consistent with the findings of the PBMC cultures (Figure 2A). These infections utilized a single donor pool, were performed in duplicate, and were repeated with consistent results. PBMCs of all cultures remained viable for 21 days. Figure 3

illustrates the relationship between persistent infection and input GBV-C concentration. Of note, the amount of input GBV-C RNA did not correlate well with persistent replication, for example patient 10 demonstrated persistent infection despite a plasma GBV-C RNA concentration 60-fold less than that of patient 4, whose GBV-C did not persist in culture (figure 3). To determine if the GBV-C replication observed for these 12 isolates in PBMC culture was independent of the input GBV-C RNA, plasma obtained from the 12 GBV-C/HIV coinfected people was normalized and used to infect PHA, IL-2 stimulated HD PBMCs from a single donor in duplicate (MOI = 0.01 GBV-C RNA genome equivalents). GBV-C RNA was not identified in culture supernatant fluids 14 days post-infection for patients 1,2,3,6, and 11 (data not shown), generally consistent with the non-normalized infections.

Pooled HD PBMCs were also infected with plasma from an HIV-negative, GBV-C RNA positive subject (#13; the source patient used to prepare the infectious clone) and culture supernatants derived from cells initially transfected with RNA transcribed from the infectious clone AF121950. The clinical isolate (#13) and virus derived from the infectious clone (#14) replicated for only $\leq$ 14 days (range 3 – 14; figure 4). In contrast, PBMCs from patient 12 demonstrated replication for more than 60 days.

GBV-C isolates from culture supernatant fluids (20 μl inocula) were serially passaged 4 times into HD PBMCs. Culture supernatants from each patient's last PBMC culture that tested positive for GBV-C RNA served as the inocula. PBMC cultures were maintained for 2 weeks, with fresh HD PBMCs added 7 days post-infection. Serial passage of GBV-C from patients 1, 7, and 11 was not done due to insufficient culture supernatant material. All 9 patients who had supernatant available for passage

demonstrated replication in PBMC cultures at the end of 4 passages, and GBV-C replication and persistence appeared improved when compared with initial PBMC cultures (Figure 5). For example, patients 5 and 6 had GBV-C RNA in passaged culture supernatant for 14 days, compared to only 5 days in the original PBMC culture.

To determine if selection for specific minor quasispecies in the initial plasma inocula replicated preferentially in PBMC culture, a 194 base-pair segment of the NS5A sequence homologous to the putative interferon-sensitivity determining region of HCV was amplified from both the initial plasma sample and from the last GBV-C RNA-positive coculture supernatant sample from patients 2, 4, 5, and 8. A minimum of five clones from each sample were sequenced. The NS5A sequence of patients 2, 4, and 5 was the same in the plasma-derived GBV-C and in the culture supernatant-derived GBV-C. In contrast, patient 8 demonstrated selection of a distinct quasispecies containing 14 mutations in all 5 clones sequenced (figure 6). These mutations did not result in a predicted amino acid change in NS5A; however, the data are consistent with the selective replication of a minor quasispecies in PBMC culture, as has been described previously (Fogeda, et al. 2000).

To determine if PHA – IL-2 stimulation of PBMCs was required for GBV-C replication in PBMC cultures, PBMCs from patient 12 were cultured with and without IL-2 and PHA. In addition, serum from patient 12 was used to infect HD PBMCs which were then cultured with and without IL-2 and PHA. GBV-C RNA in culture supernatant from day 7 was quantified by real-time PCR. Figure 7 demonstrates that the amount of GBV-C RNA detected in supernatant was significantly greater in both serum-infected

HD cells and infected patient PBMCs when IL-2 and PHA were not added to culture media.

To evaluate HIV replication in the PBMC cultures, HIV p24 antigen was measured in supernatants obtained from cocultured patient PBMCs and infected HD PBMCs on day 14. No HIV p24 antigen was detected in supernatants of infected HD PBMCs, however HIV p24 antigen was detected in supernatants from PBMC cocultures of patients 4, 6, and 10 (data not shown). Patients 4 and 10 both had detectable HIV in plasma on the day of PBMC collection (Table 2).

Sequence analysis of a GBV-C isolate that persists in PBMC culture: As GBV-C RNA from patient 12 demonstrated persistent replication in multiple PBMC cultures, we determined the entire nucleotide sequence of this isolate. A series of nested sense and antisense oligonucleotide primers were designed to span the entire open reading frame from nt 41 to 9349 (sequence numbers based on GBV-C isolate # AF121950; Genbank) using a strategy similar to that previously described (Xiang, et al. 2000). PCR products were purified, ligated into the pTA vector, and the sequence was determined as described above. At least 5 clones of each PCR product were sequenced to determine the consensus sequence (GenBank accession number AY196904).

The sequence contained a long open reading frame (ORF) that began at nt 351 (using sequence numbering of AF121950) and extending to nt 9080. This ORF is predicted to encode a 2,974 amino acid polyprotein with a molecular weight of 313,788 daltons. If translation initiates upstream of the amino terminus of the predicted E1 protein (Simons et al. 1996), the polyprotein would be 2,906 amino acids (MW 312,989 daltons). There are 4 AUG codons in frame with the polyprotein upstream of the

putative translational start site of GBV-C in this isolate which could potentially encode a core protein. The complete GBV-C sequence of this isolate was compared with 20 full length human isolates obtained by searching GenBank for complete GBV-C sequences (GenBank access numbers: AF121950, AB 320090, AB 320091, AB 320092, AB 003293, AB 008335, D90600, D 90601, D 87255, D 87262, D 87708, D 87709, D 87710, D 87711, D 87712, D 87713, D 87714, D 87715, U 44402, U 63715). Nucleotide and predicted amino acid sequences were aligned, and the evolutionary distance between sequences was determined using the Jukes-Canter method (DNAMAN software, Lynnon BioSoft Inc, Quebec, Canada). Comparison of the polyprotein sequence of these isolates revealed a 96.53% homology in the polyprotein coding sequences (Figure 8). Sixteen of these sequences (all except AB320090, AB320091, AB320092, and AB320093) contained the complete 5'ntr and 3'ntr sequence, allowing comparison of full-length genome nucleotide sequences. Comparison of these isolates revealed 89.54% homology at the nucleotide level (data not shown).

A consensus predicted amino acid sequence was generated from these 21 GBV-C polyproteins (initiating translation at nt 554 of AF121950). There were 62 amino acids differing between our isolate and the consensus sequence (2.9%). Based on the predicted protein encoding regions of the genome, most of the differences between our sequence and the consensus sequence occurred in the NS2 and ES2 coding regions (4.6% and 4.4%, respectively), followed by the NS5A region (3.6%), NS4 (2.5%), NS5B (2.1%), E1 (1.6%), and NS3 (1.2%). There were 16 amino acid polymorphisms identified in our isolate that are not present in any reported GBV-C sequence. Fourteen

of these were in nonstructural proteins NS5A (6), NS5B (4), and NS3 (4) as seen in figure 9A.

Since AY196904 (patient 12) reproducibly demonstrated prolonged replication in PBMC cultures, and AF121950 isolate did not, we compared the predicted amino acid sequence for the polyproteins of these two isolates. There was 97.6% homology between the two isolates, and the region of greatest divergence was E1 (5.3%) followed by E2 (3.6%), NS2 (3.2%), NS4 (1.9%), NS5A (1.7%), NS5B (1.6%), and NS3 (1.3%). Of note, the NS5B substitutions I→V at 2465 and A→G at 2795 are very similar to mutations in HCV NS5B that are associated with increased RNA replication in HCV RNA replicon systems (Lohmann et al. 2001); Figure 9B). Specifically, HCV substitutions I2442V and R2884G were found to result in a 500-fold increase in RNA replication (18). In addition, the substitutions 2070-2071 VS→MT and 2074 I→L are found in the region homologous to the putative interferon-sensitivity determining region of HCV (Fujisawa et al. 2000;Kato et al. 1999), and are not found in any other GenBank sequence (Figure 9C). We previously identified similar substitutions (2069-2071 EVS→KMT) in an interferon-sensitive GBV-C clinical isolate (Xiang et al., manuscript submitted), suggesting that this region may be important in evasion of natural host antiviral defense mechanisms. NS5A is thought to be phosphorylated in HCV (Reed & Rice 1999; Reed, et al. 1997). The mutations noted in Figure 9C would potentially alter phosphorylation at amino acids 2071 and 2151.

DISCUSSION

GBV-C replication has been associated with decreased HIV replication *in vitro* (Xiang, et al. 2001) and *in vivo* (Tillmann, et al. 2001; Xiang, et al. 2001). In our experience, GBV-C *in vitro* replication methods were inefficient and inadequately reproducible, even using an infectious molecular clone. To better characterize GBV-C replication, we employed three different PBMC-based culture methods to measure persistence of GBV-C replication in culture. Clinical isolates propagated either from GBV-C infected patient PBMCs or donor PBMCs infected with GBV-C RNA positive plasma demonstrated two growth patterns. Seven of the 12 clinical isolates demonstrated GBV-C growth in culture supernatants for < 7 days, while the other 5 isolates consistently persisted in culture for >14 days (Figure 2). With two exceptions (patients 4 and 11), the growth pattern was consistent when cultures were normalized for input GBV-C RNA. Coculture of GBV-C positive PBMC's with donor PBMCs did not improve GBV-C persistence, and GBV-C culture in media supplemented with PHA and IL-2 inhibited growth (Figure 7). Since the 12 GBV-C isolates persisted for similar duration in PBMC cultures from either pooled or single donors on multiple occasions, it appears that the capacity to persist in PBMC cultures is an intrinsic viral characteristic. Nevertheless, host-cell factors may also contribute to this persistence.

Replication of GBV-C in these 3 culture systems was not associated with cell toxicity, as significant cell death was noted in only one patient's PBMC cultures. GBV-C replication was independent of the source patient's CD4+T cell count, CD8+T cell count, hepatitis C antibody status, gender, age, or HIV acquisition mode. High HIV RNA levels in detected in patients 3 and 4 may explain the relatively poor GBV-C replication of

these isolates. However, when GBV-C from patient 3 was tested for growth in HD PBMCs at a time with the source patient's HIV RNA was <400, no improvement in GBV-C growth was noted, and GBV-C from patient 10 consistently grew well in culture despite ongoing detectable HIV RNA (data not shown).

GBV-C replication in PBMC culture did not correlate with plasma GBV-C RNA titer (Figure 3), and growth patterns remained distinctive when GBV-C RNA input was normalized. The twelve patients studied had a broad range of GBV-C RNA concentrations, ranging from 2.3×10^4 GE/mL to 2.5×10^8 GE/mL. These data indicate that the range of GBV-C RNA concentrations in patient plasma varies more widely than either HIV or HCV (Tillmann, et al. 2001). The relationship between GBV-C RNA concentration and HIV replication requires further study.

Serial passage of culture supernatant fluids into donor PBMCs appeared to result in prolongation of GBV-C replication in PBMCs (figure 5). Improved replication of GBV-C passaged from culture supernatant fluids into donor PBMCs may represent selection of viral quasispecies more adapted for *in vitro* replication. Consistent with this, we isolated a distinct GBV-C quasispecies in culture supernatants from patient 8 that was not identified in patient plasma prior to culture (figure 6), as noted by others (Fogeda, et al. 2000). Studies are underway to further adapt GBV-C to growth in cell culture systems.

In the course of these studies, we noted that GBV-C from patient 12 consistently demonstrated prolonged replication in culture. This isolate persisted in PBMC culture longer than GBV-C from an HIV negative patient, and when compared with our previously described infectious clone. The full-length sequence of the polyprotein

coding region of this isolate (AY196904) revealed 97.6% homology with the predicted polyprotein consensus sequences of 20 other full-length GBV-C genomes in GenBank. Sixteen unique amino acid substitutions were found, clustered in the key replication nonstructural proteins NS3, NS5A, and NS5B (figure9A). This GBV-C isolate contained amino acid substitutions in both NS5A and NS5B coding regions similar to changes identified in HCV NS5A and NS5B previously found to be involved in interferon sensitivity and enhanced RNA replication (Blight, et al. 2000; Gale et al. 1998; Krieger, et al. 2001). Thus, amino acid substitutions in key GBV-C proteins may play critical roles in viral replication and host immune evasion. We are constructing chimeric virsuses using AF121950 and AY196904 sequences to further explore this possibility.

In summary, GBV-C in the plasma of individuals with HIV co-infection demonstrated different replication phenotypes in PBMC-based cultures *in vitro*. No improvement in GBV-C replication was noted when cultures were supplemented with donor PBMCs, and GBV-C replication was decreased when IL-2 and PHA were added to PBMC cultures. Persistence of GBV-C replication in PBMCs was not correlated with clinical features of the source patient, including plasma GBV-C RNA concentration or the presence of E2 antibody. Specific nucleotide sequence mutations were identified following two weeks in culture in one isolate, suggesting that specific GBV-C nucleotide polymorphisms may influence GBV-C *in vitro* replication. The full-length polyprotein sequence of a GBV-C isolate that consistently demonstrated prolonged replication in culture also contained unique amino acid substitutions in non-structural proteins involved in viral replication and evasion of the host immune response. Given the *in vivo* and *in vitro* evidence that GBV-C replication is associated with inhibition of HIV

replication, it is hoped that these studies may allow the development of more robust models for studying GBV-C – HIV interactions.

Methods

Study participants: Individuals attending the University of Iowa HIV clinic between 1999 and 2002 were invited to participate in a study of GBV-C infection. In addition, an HIV-negative, GBV-C RNA positive person and several healthy individuals (who were negative for HIV, HCV and GBV-C) participated in these studies. Informed consent was obtained from all participants and the study was approved by the University of Iowa Institutional Review Board. Medical records were reviewed to confirm HIV results and related clinical information. HIV-RNA and CD4+T cell counts were obtained within 30 days of blood collection for these studies, and ALT levels were obtained within 90 days. HCV antibody testing was performed during the initial clinic visit. All statistics were performed using SigmaStat software V2.03S (Jandel Scientific, Chicago, IL).

GBV-C RNA Detection and Sequence Determination: Blood samples were anticoagulated (acid-citrate-dextrose; Becton-Dickinson, Franklin Lakes, NJ) and processed within 2 hours of collection. Whole blood or plasma (200 μl) was added to 500 μl GITC solution and stored at –80°C as previously described (Schmidt et al. 1995; Xiang et al. 1998). RNA was extracted as previously described (George et al. 2002a), and the final RNA quantity used as template in each RT-PCR reaction represented 50 μl of the original sample. Primers from the GBV-C 5' non-translated region (5'ntr) of GBV-C (nt #s 23-330) were used in nested RT-PCR reactions as previously described (Schmidt et al. 1997b; Schmidt et al. 1997a; Stapleton et al. 1999;Xiang, et al. 1998). In addition, primers from the GBV-C NS5A region (nt#s 6651-6877) were used to amplify viral RNA: (outer antisense: TACTGCARTCYTCCATGATGACAT; outer sense: ATGGTYTAYGGYCCTGGVCAAA; nest antisense: TTCAAGAATCCTCGCAGCATTCT;

nest sense: CTGGVCAAAGYGTYACCATT). All sequence numbers are based on the sequence of the infectious GBV-C clone, GenBank Accession #AF121950 (Xiang, et al. 2000). At least 2 negative and positive control samples were evaluated with each PCR reaction, and all samples and controls were tested in duplicate. Where noted, RNA extracted from culture supernatant fluids (50 μl) and PBMCs (5×10^5) was tested for GBV-C RNA in nested RT-PCR reactions as previously described (George, et al. 2002a; Xiang, et al. 2000). To determine GBV-C genotype, first round products from 5'ntr RT-PCR were amplified using primers specific for GBV-C genotypes I-IV as described by Naito et al (Naito & Abe 2001). PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

GBV-C PCR products were purified (Wizard PCR purification kit, Promega, Madison, Wis.) and ligated into the pTA vector (Original TA cloning kit; Invitrogen, Carlsbad, Calif.) as previously described (Stapleton, et al. 1999). Plasmid DNA was sequenced using an ABI automatic sequencer (University of Iowa DNA Core facility; (George, et al. 2002a). Sequences were analyzed using DNAMAN software (DNAMAN software, Lynnon BioSoft Inc, Quebec, Canada).

Culture Methods and Infectivity Assays: PBMCs from GBV-C/HIV coinfected people and healthy donors (HD PBMCs) who were HIV, HCV, GBV-C negative were prepared by Ficoll-Hypaque followed by centrifugation as previously described (Cook et al. 1997). PBMCs obtained from GBV-C infected donors were maintained in stimulation media (RPMI 1640 media supplemented with 10% FCS, 5% recombinant human IL-2 (Cellular Products Inc., Buffalo, N.Y.), 10 mcg/mL phytohemagglutinin (PHA, Difco, Detroit, MI), and 10 mcg/mL lipopolysaccharide (*Escherichia coli* LPS, Sigma) at a concentration of

2 x10^6 PBMCs/mL for 48 hrs. (Xiang, et al. 2000). PBMCs were then incubated in RPMI 1640 media supplemented with 10% FCS, 5% recombinant human IL-2, and 5 mcg/mL PHA at a concentration of 2 x10^6 PBMCs/mL, and media was changed weekly. For co-culture experiments, an equal number of PHA-IL-2 stimulated HD PBMCs were added when PBMCs from GBV-C infected donors were obtained and weekly thereafter. For infectivity experiments, HD PBMCs were stimulated for 48 hours prior to infection with GBV-C or mock serum, and maintained as above.

GBV-C replication and persistence were studied in three ways (Figure 1). First, PBMCs from GBV-C/HIV coinfected people were cultured without donor cells. Secondly, PBMCs from the same people were cultured with the addition of an equal number of HD PBMCs and fresh HD PBMCs were added weekly. The same PBMC donor pool was used for each culture and each weekly supplementation. Finally, plasma from the same GBV-C RNA/HIV coinfected people was used to infect PHA, IL-2 stimulated donor PBMCs. The same PBMC donor pool was used for each infection, and plasma infections of all 12 clinical isolates were done simultaneously. PBMCs ($5x10^5$) were removed weekly and stored in GITC at -80°C until use, and culture supernatants (500 µl) were removed twice weekly and stored at –80°C. Mock control infections of donor PBMCs were maintained under identical conditions.

GBV-C RNA quantitation: GBV-C RNA was purified from plasma, PBMCs or culture supernatant and quantified using both real-time PCR (Perkin-Elmer, Branchburg, N.J.) and endpoint dilution using nested RT-PCR. RNA from 100 µl plasma was used as the template for RT-PCR using primers from the 5'ntr region described above. The product of the RT-PCR was used as the template for real-time PCR with primers from the 5'ntr

region (sense primer at nt 211: TACCGGTGTGAATAAGGGCC; antisense primer at nt 283: CGTCGTTTGCCCAGGTG), a 6-FAM/TAMRA labeled probe corresponding to nt # 241-265 (CTCGTCGTTAAACCGAGCCCGTCAC), and TaqMan Universal PCR Master Mix (Perkin-Elmer, Branchburg, N.J.). Samples were incubated at 50°C for 2 minutes, followed by 95°C for 10 minutes, then amplified in 40 cycles (50°C for 15 seconds and 60°C for 1 minute) in an ABI Prism 7700 sequence detector. Duplicate positive and negative controls were performed with each reaction. A standard curve was determined using terminal dilution PCR to relate cycle threshholds (Ct) and genome equivalents (GE) of GBV-C RNA per mL of plasma or supernatant (adjusted R^2=0.985; $p<0.001$; SigmaStat). Real-time PCR results were compared with terminal dilution experiments using RT-PCR. Terminal dilution experiments utilized $\log_{10}$ serial dilutions of RNA as the template for RT-PCR, and PCR was performed using primers from two regions of the genome (5'ntr and NS5A).

HIV p24 antigen assay: Culture supernatants fluids obtained two weeks post-infection (450 μl) were analyzed in duplicate for HIV replication using Retro-Tek HIV-1 p24 antigen ELISA kits (Zeptometrix, Buffalo, NY) as previously described (Wuenschmann & Stapleton 2000; Xiang, et al. 2001). Duplicate positive, negative, and substrate controls were run with each assay and a p24 antigen standard curve from 0 to 125.0 pg/mL was prepared in accordance with the manufacturers instructions.

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Figur 1: Culture methods used to characterize GBV-C replication. Plasma and PBMCs were isolated from GBV-C/HIV infected people by standard methods and PBMCs were cultured alone (A) or supplemented with an equal number of healthy donor PBMCs (B). Alternatively, GBV-C positive plasma was used to infect healthy donor PBMCs (C). Culture supernatants and cells were collected for GBV-C RNA analysis.

Figure 2: GBV-C clinical isolates were analyzed for infectivity using three modalties. The mean duration of persistence for all expereiments is shown, along with the minimum (solid boxes) and maximum (open boxes) duration of GBV-C RNA detection in culture supernatants for all three culture modalities (A). PBMCs were cultured without (B) or with (C) supplementation with an equal number of donor PBMCs. Insufficient cells were available for PBMC infection for subject 8. nt = not tested. The GBV-C plasma RNA titer (GE x 10^5/mL plasma) for each patient is shown beneath the X axis.

Figure 3: Correlation between input GBV-C RNA concentration and maximum time of persistent infection. . Plasma GBV-C RNA concentration in 20 μL from lowest to highest is presented on the X axis.

Figure 4: GBV-C persistence of clinical isolates. GBV-C from an HIV-negative source patient (#13) and from virus derived from the GBV-C infectious clone (#14) were used to infect healthy donor PBMCs and compared with plasma GBV-C from patients 8 and 12. The clone was prepared from patient 13 (GenBank # AF121950). **GBV-C from patient 12 persisted in PBMC cultures for 60 days (data not shown).

Figure 5: Serial passage of GBV-C from PBMC coculture supernatant into donor PBMCs. The same donor pool was used for each passage, and all cultures were maintained for 14 days. Supernatant from patients 1, 7, and 11 were not available for testing. Total days GBV-C RNA was detected in culture supernatant after 4 passages is shown for each patient.

Figure 6: Selection of GBV-C nucleotide mutations in supernatant on day 14 (patient 8). A segment of the NS5A region is shown (Panel A = 1-97, Panel B = 98-194). The nucleotide sequence of the plasma GBV-C isolate is shown (4 clones) and compared to the sequence obtained from day 14 culture supernatant (5 clones).

Figure 7: Peripheral blood mononuclear cells (PBMCs) from a GBV-C/HIV infected patient (patient 12; G+ donor) and healthy donor PBMCs infected with patient serum from patient 12 (G- donor) were cultured with and without PHA/IL-2 supplementation in media for 14 days. GBV-C RNA concentrations measured in day 7 culture supernatant are shown. The results were consistent at day 14.

Figure 8: Comparison of the predicted full-length polyprotein sequence of the isolate from patient 12 (GenBank #AY196904) with predicted polyprotein sequences of 20 full-length GBV-C RNA sequences. The phylogenetic tree was created using the Jukes-Cantor method and percentage homologies of the polyprotein sequences are shown. Isolates AY196904 (patient 12) and AF121950 (infectious clone) are highlighted, as these are the only isolates characterized for replication in PBMC culture.

Figure 9: Amino acid polymorphisms in AY196904. Sixteen amino acids were identified in AY196904 that were not present in the other sequences (A). Comparing the isolate from patient 12 (AY196904) with the infectious clone (AF121950), 2 amino acid differences were identified in the NS5B region (panel B) similar to those related to RNA replication in HCV (Lohmann, Korner, Dobierzewska, & Bartenschlager 2001). Similarly, comparison of predicted NS5A amino acid sequences from AY196904 and AF121950 revealed differences in a region homologous to the HCV interferon-sensitivity determining region (panel C).

Table 1. Clinical features of GBV-C/HIV coinfected patients studied.

Patient	CD4 Cells*	CD8 cells*	HIV RNA**	HCV antibody***	ALT
1	251	1086	<400	Pos	57
2	161	540	<400	Neg	22
3	64	654	233,000	Neg	48
4	127	928	69,000	Pos	147
5	197	1942	<400	Neg	19
6	443	1894	<400	Neg	23
7	254	1129	<400	Neg	16
8	701	1336	<400	Neg	19
9	885	2588	<400	Pos	30
10	113	1806	20,000	Neg	25
11	222	1126	<400	Neg	11
12	247	856	500	Neg	10

*CD4 and CD8 cells = number of cells/mm^3, **HIV RNA = Genome equivalents/mm^3

***Pos. = positive, Neg. = negative, ALT= alanine amino transferase, IU/mL.

Table 2. Characteristics of GBV-C viremia and E2 antibody in 12 patients with GBV-C - HIV co-infection.

	Plasma GBV-C titer		
Patient	Real-Time PCR	Quantitative PCR (Td)	GBV-C Genotype
1	2.97×10^{5}*	2.5×10^{5}	2
2	9.5×10^{5}	2.5×10^{5}	2
3	2.25×10^{6}	2.5×10^{6}	1,2
4	5.86×10^{6}	2.5×10^{6}	2
5	2.2×10^{4}	2.5×10^{4}	2
6	9.26×10^{5}	2.5×10^{5}	2
7	5.65×10^{5}	2.5×10^{5}	2
8	1.16×10^{8}	2.5×10^{8}	2
9	5.6×10^{8}	2.5×10^{8}	2
10	1.19×10^{5}	2.5×10^{5}	2
11	1.60×10^{6}	2.5×10^{5}	2
12	4.26×10^{8}	2.5×10^{8}	2

All data represent GBV-C RNA genome equivalents/mL of plasma determined in nested RT-PCR reactions. Td = semi-quantitative PCR determined by terminal dilution of input RNA.